

1 Method of Peptide Synthesis

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3 The chemical synthesis of peptides up to 40
4 residues is now routinely efficient and recent
5 advances over the last 10 years has led to the
6 synthesis of peptides and small proteins in the
7 range of 40-150 residues. Efficient novel
8 synthetic methodology and a wide array of resins
9 which can be used for synthesis have contributed to
10 this.

11

12 One particular resin, developed by Wang, S.S.
13 *J.Amer.Chem.Soc.* **95**, (1973), 1328, (see figure 1)
14 has become the industry standard which has proven
15 effective in the efficient synthesis of long
16 peptides. There are however a number of problems
17 with this resin which relate to the C-terminal
18 amino acid. Firstly esterification of the resin
19 with protected derivatives of cysteine and
20 histidine can cause significant levels of
21 racemisation which, of course, is highly
22 undesirable. Further, whilst esterification with

1 protected derivatives of proline is successful
2 problems are encountered after an additional amino
3 acid residue is added to form a dipeptide.
4 Deprotection of the dipeptide in preparation for
5 the coupling of the third amino acid gives a free
6 amino dipeptide ester which often cyclises
7 internally to form the free cyclic dipeptide (a
8 diketopiperazine) shown in figure 2. The resultant
9 loss of dipeptide is in most cases quantitative and
10 renders use of the Wang resin unsuitable for the
11 synthesis of C-terminal proline peptides. Moreover
12 it has also been suggested that cyclisation also
13 occurs when the penultimate C-terminal residue is a
14 proline residue or one of its derivatives.

15
16 The use of the sterically hindered and extremely
17 acid labile 2-chlorotrityl chloride resin (see
18 figure 3) is recommended for the synthesis of C-
19 terminal proline containing peptides (as the steric
20 bulk inhibits diketopiperazine formation).

21
22 Experiments were carried out to synthesise medium
23 length and long peptides where, due to the nature
24 of the C-terminal residue, 2-chlorotrityl resin was
25 used. The medium length peptide (about 30 residues)
26 was HNP-1 where the C-terminal residue is cysteine
27 The long peptide was guinea pig eotaxin, a 74 amino
28 acid peptide, of which the C-terminal residue is
29 proline.

30
31 Both experiments were unsuccessful. Low yields of
32 both peptides were obtained and monitoring of the

1 chain assembly showed a low coupling efficiency in
2 both cases. By comparison with the situation when
3 the HNP-1 peptide was synthesised on a Wang resin
4 using a resin loading procedure that was reported
5 to alleviate the problem of racemisation of C-
6 terminal cysteine, the chain assembly proved
7 excellent and the low yield obtained with the
8 chlorotrityl resin was ascribed to some property of
9 that resin.

10

11 One theory was that the extreme acid lability of
12 this resin led to a premature cleavage of the
13 peptide from the resin during chain assembly. The
14 inventors varied the conditions of synthesis to try
15 to eliminate the contact of the resin with acid
16 species during chain assembly of guinea pig eotaxin
17 but no improvement in yield was achieved. Another
18 theory is that some property of the 2-chlorotrityl
19 resin, e.g. swelling characteristics, renders it
20 unsuitable and inefficient in the assembly of long
21 peptides.

22

23 Thus 2-chlorotrityl resin appears only compatible
24 with the synthesis of relatively short (e.g. <20
25 residues) peptides. It has now been found that the
26 problems associated with respect to a peptide
27 containing a C-terminal proline on 2-chlorotrityl
28 resin can be alleviated if the synthesis is carried
29 out on the Wang resin.

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1 **Summary of the Invention**

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3 The invention relates to a method for synthesis of
4 a given peptide which contains a proline or one of
5 its derivatives, at proximity to, or at, the C-
6 terminus end of the peptide of interest. This
7 method is particularly suitable for the synthesis
8 of long peptides, for example peptides which have
9 at least 20 amino acid residues or for peptides
10 where synthesis is problematic on 2-
11 chlorotritylchloride resin.

12

13 By the expression "proximity to" it is meant that
14 the proline residue is positioned at the
15 penultimate C-terminal position.

16

17 The expression "derivatives" is directed to a
18 peptide, an amino acid or an amino acid residue
19 which may differ from the corresponding peptide
20 amino acid or residue by the substitution/addition
21 of various substituents. It is usual in protein
22 synthesis to use modified amino acids having
23 protecting groups or which have been modified so as
24 to be able to act as labels or tags or for other
25 desirable purposes. For example, in the method of
26 the present invention amino acid derivatives such
27 as hydroxyproline or other proline derivatives
28 could be used.

29

30 In a preferred embodiment, the method comprises the
31 steps of:

- 1 a) synthesising on a first resin a C-
2 terminal portion of said peptide, or its
3 derivative, comprising at least three
4 successive amino acid residues or their
5 derivatives, by successive coupling of
6 selected amino acids, small peptides or
7 their derivatives, said first resin being
8 suitable for the formation of peptides
9 having a proline residue or a proline
10 derivative positioned at, or at proximity
11 of, the C-terminal end of said peptide;
12 b) cleaving the C-terminal portion thus
13 obtained from said first resin;
14 c) reattaching said C-terminal portion to a
15 second resin which is generally suitable
16 for the synthesis of peptides but is
17 unsuitable for the formation of peptides
18 having a proline residue or a proline
19 derivative positioned at, or at proximity
20 of, the C-terminal end of said peptide;
21 and
22 d) coupling selected amino acids, small
23 peptides or derivatives to the C-terminal
24 portion to obtain said given peptide.

25
26 Whilst peptides of any length can be synthesised
27 using the method of the invention, the method is
28 particularly suited for the synthesis of peptides
29 having at least 20 amino acid residues or "long
30 peptides". The method is particularly suitable for
31 peptides having up to about 150 amino acid
32 residues.

1 The method of the invention allows synthesis of
2 peptides which were otherwise difficult to obtain
3 quantitatively. Amongst such peptides which have a
4 C-terminal proline residue and can be obtained
5 using the method of the invention chemokines are of
6 particular interest and particularly the human
7 chemokines IP-10, BLC and MCP-2.

8
9 Advantageously, the first resin is chosen so that
10 it does not lead to the formation of cyclic
11 dipeptides and in particular to the formation of
12 diketopiperazine compounds.

13
14 Step a) and/or d) of the method of the invention
15 may be achieved by successive coupling of the
16 predetermined amino acid residues, small peptides
17 or their derivatives. This can be carried out
18 using standard solid phase procedures which are
19 well known. In these procedures, the α -amino group
20 of the next selected amino acid or small peptide is
21 protected using a protecting group and is added to
22 the resin bearing the C-terminal portion of the
23 peptide together with a coupling agent like
24 diisopropylcarbodiimide (DIC) or
25 dicyclohexylcarbodiimide (DCC). The α -amino
26 protecting group is then removed by exposure to a
27 suitable base which leaves the peptide bond intact
28 and the next amino residue can then be added by
29 repeating the above step. Such procedures are
30 detailed for example in W.C. Chan and P.D. White,
31 Fmoc Solid Phase Peptide Synthesis A Practical
32 Approach, OUP 2000.

1 A preferred first resin for the formation of the C-
2 terminal portion is the 2-chlorotrityl chloride
3 resin or any similar resin which inhibits or
4 minimises the formation of diketopiperazine.

5
6 A preferred resin to be used as the second resin
7 for synthesis of a long peptide which can be used
8 in the method of the invention is a resin having
9 benzyl ester linker like the 4-(3-methoxy-4-
10 (hydroxymethyl)phenoxy-methyl) derivative of
11 polystyrene-co-divinylbenzene which is marketed
12 under the Trade Mark SASRIN™. A particularly
13 preferred resin is a 4-Hydroxymethylphenoxy-methyl
14 resin known as Wang resin. Wang resins are well
15 known and widely available.

16
17 Advantageously, the cleaving step from the first
18 resin is achieved using a mild acid treatment, for
19 example 20% trifluoroethanol in dichloromethane.
20 This allows a fully protected (tri-) peptide moiety
21 to be obtained. Thus, the C-terminal portion can
22 be provided fully protected so it can be coupled
23 directly onto the resin suitable for synthesis of a
24 long peptide. The protective groups may be the
25 standard protective groups usually used in Fmoc (9-
26 fluorenylmethoxycarbonyl), Nsc (2-(4-
27 nitrophenylsulfonyl)ethoxycarbonyl) or t-Boc (ter-
28 butyloxycarbonyl) peptide synthesis.

29
30 The invention will now be described by way of
31 example only, with respect to figures in which:

32

1 Figure 1: shows molecular structure of the Wang
2 resin linker.

3 Figure 2: shows formation of diketopiperazine.
4

5 Figure 3: shows molecular structure of the 2-
6 chlorotrityl chloride resin linker.
7

8 **Example**

9
10 The synthesis of guinea pig eotaxin, which contains
11 a C-terminal proline residue, has been achieved
12 using this resin exchange technique with an overall
13 yield of 5mg following purification and disulphide
14 bond formation. When one considers that the same
15 scale synthesis performed on a 2-chlorotrityl resin
16 typically yields < 1mg overall, the advantages of
17 the method according to the invention are clearly
18 evident.
19

20 Any protein/peptide susceptible to diketopiperazine
21 formation can be assembled using this described
22 strategy. Polypeptides or proteins that contain
23 proline or proline derivatives at, or adjacent to,
24 the C-terminus are susceptible to diketopiperazine
25 formation during assembly. The described approach
26 will be extremely enabling for the synthesis of
27 such peptides.
28

29 Synthesis of gp eotaxin protected C-terminal
30 tripeptide on 2-chlorotrityl resin (Fmoc-Thr(Bu^t)-
31 Lys(Boc)-Pro-ClTrtR) (1)
32

1 Peptide synthesis was carried out on the ABI 430A
2 peptide synthesiser. H-Pro-2-chlorotrityl resin
3 (1g, 0.49mmol/g, Lot no. PrT-2, Nankai Hecheng Co.
4 Ltd., China) was used in the reaction vessel. Nsc-
5 Lys(Boc)-OH (503mg, 1mmol) was activated with HOCT
6 (4ml, 1mmol, GL Biochem, (Shanghai) Ltd. China) and
7 DIC (4ml, 1mmol, Acros) for 15mins then transferred
8 to the reaction vessel and coupled for 30mins. A
9 second cartridge of Nsc-Lys(Boc)-OH was activated
10 similarly and recoupled to the resin after draining
11 the first solution.

12

13 Following capping of unreacted amino groups on the
14 resin with acetic acid anhydride (0.5M in DMF,
15 10ml) the Nsc group was removed with Deblock
16 solution (1% DBU, 20% piperidine in DMF).

17

18 Fmoc-Thr(But)-OH (397mg, 1mmol, Applied Biosystems)
19 was activated in the same manner and coupled to the
20 resin for 30mins followed by recoupling of the same
21 amino acid as before. After coupling the resin was
22 washed with DMF then DCM and dried under vacuum
23 giving a yield of 1.21g of (1).

24

25 The synthesis was repeated using a further gram of
26 resin furnishing 1.18g of the title resin. The
27 resin batches were combined for further work.

28

29 Cleavage and isolation of Fmoc-Thr(Bu^t)-Lys(Boc)-
30 Pro-OH (2)

31

1 The peptide resin (1) was stirred in a solution of
2 trifluoroethanol (20%) in DCM (50ml) for 60mins.
3 The resin turned dark green. The solution was
4 filtered and evaporated under reduced pressure to
5 give an oil which was triturated with cold diethyl
6 ether / hexane. The solvent was evaporated and
7 fresh hexane added to yield a solid from which the
8 solvent was again removed by evaporation. A white
9 solid (400mg, 0.55mmol) was obtained. Mass
10 spectroscopy Electrospray positive ion found 723.4,
11 expected for $C_{39}H_{54}N_4O_9$ 722.4 kD.
12

13 Coupling of (2) to Wang resin to give Fmoc-
14 Thr(Bu^t)-Lys(Boc)-Pro-O-Wang resin (3)
15

16 The protected tripeptide (2) (400mg, 0.55mmol) was
17 dissolved in the minimum volume of DMF (<2ml) and
18 activated by the addition of DIC (86 μ l, 0.55mmol)
19 and sonicated for 15mins.
20

21 Wang resin (800mg, 0.56mmol/g, Lot no. W-34,
22 Nankai Hecheng Co. Ltd., China) was swollen in the
23 minimum volume of DMF until just freely mobile and
24 dimethylamino pyridine (a few crystals) added. The
25 activated peptide solution (2) was added and the
26 coupling reaction sonicated for 4h. The mixture
27 was then filtered and the resin washed with DMF,
28 DCM and diethyl ether successively. The resin was
29 dried under vacuum to give a final yield of 1.0g.
30 The Fmoc loading test was carried out on the resin
31 and a final loading of 0.162mmol/g was determined.
32 It was established using Izumiya test that the

1 loading of the tripeptide onto the Wang resin was
2 racemisation free.

3

4 Synthesis of gp eotaxin on Wang resin

5

6 The synthesis of gp eotaxin was carried out using
7 500mg, 0.081mmol of resin (3). Standard coupling
8 cycles using 1mmol of amino acid (HOt 2ml, 1mmol)
9 and DIC (2ml, 1mmol) were carried out on the ABI
10 synthesiser with the exception that:

- 11 a) the next amino acid Fmoc-Thr(Trt)-OH was
12 coupled without a prior capping step on
13 the resin and
14 b) the N-terminal amino acid Fmoc-His(Trt)-
15 OH was coupled using HOBt 2mmol in place
16 of HOt.

17

18 The final Fmoc group was retained on the resin as a
19 purification tag.

20

21 Cleavage, purification and isolation of gp eotaxin

22

23 After chain assembly, the Fmoc-peptide was cleaved
24 with EDT/H₂O/TIS/thioanisole/ TFA
25 (0.5/1.0/0.2/0.2/10ml) at 0°C under nitrogen for
26 4h. The resin was removed by filtration and peptide
27 precipitated into cold ether and centrifuged. It
28 was purified by G50 Sephadex gel filtration and
29 HPLC and the amino terminal Fmoc group cleaved from
30 the protein using 20% piperidine in CH₃CN/H₂O
31 (1:1). DTT was added to reduce the side chain of
32 Cys residues and the cleaved Fmoc removed by gel

1 filtration to give the pure, reduced peptide. This
2 was folded in 50mM Tris pH8.0, 5mM GSH/0.5mM GSSG,
3 and monitored by HPLC. Folding took about a week
4 to complete.

5

6 The folded peptide was purified by HPLC, to give
7 the pure, folded peptide. (Electrospray mass
8 spectrometry; Expected mass 8356.9 Da, found 8353.9
9 Da).

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